UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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INFLUÊNCIA DE NOVOS APRENDIZADOS SOBRE O PROCESSO DE REORGANIZAÇÃO DA MEMÓRIA DE MEDO CONDICIONADO

Trabalho de Conclusão de Curso apresentado ao Instituto de Biociências da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do grau de Bacharel em Ciências Biológicas.

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Porto Alegre, 2010.

PREFÁCIO

O tema central deste trabalho de conclusão de curso é a **consolidação sistêmica da memória**, definida como um longo processo de reorganização através do qual a evocação das memórias gradualmente torna-se independente do hipocampo e passa a requerer estruturas corticais.

Esse projeto foi essencialmente inspirado pelo trabalho dos professores Jorge Quillfeldt e Ivan Izquierdo de 1996 que me instigou a estudar esse interessantíssimo fenômeno que é a reorganização da memória. Foi todo realizado no Laboratório de Psicobiologia e Neurocomputação (LPBNC) da UFRGS, sob a orientação do Prof. Dr. Jorge Quillfeldt e co-orientação do Dr. Felipe Diehl no período de 2009-2010.

Logo após uma breve introdução ao assunto abordado e ao projeto, veremos então o trabalho experimental no formato de artigo científico (pag. 13), com a intenção de ser submetido à revista *Journal of Neuroscience* (http://www.jneurosci.org/) como *Brief Comunication*.

"A escola é o sistema através do qual a sociedade se livra das crianças perguntadoras. Os que sobrevivem a esse processo são chamados de cientistas."

Herman Bondi

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1. RESUMO

Objetivos: Após uma codificação inicial, a memória passa por um lento processo de reorganização e progressivamente torna-se independente do hipocampo, passando a ser armazenada difusamente no córtex. Esse fenômeno é conhecido como Consolidação Sistêmica da memória e, apesar do grande número de evidências, ainda não é totalmente compreendido. Nossos objetivos foram investigar quanto tempo uma memória de medo condicionado leva para tornar-se independente do hipocampo (Experimento 1) e se a aquisição de novas memórias durante este período pode interferir com esse processo (Experimento 2).

Métodos: Ratos Wistar machos foram treinados na tarefa de Condicionamento Aversivo ao Contexto (3min - 2 choques 0,7mA - 1min) e testados 1, 35 ou 45 dias depois, no Experimento 1, e 20 dias depois no Experimento 2. No teste, em ambos os experimentos, os animais foram infundidos com Muscimol (1ug/lado), droga classicamente amnésica, ou seu Veículo (TFS) no hipocampo dorsal (15min pré-teste). As respostas de medo ("freezing") foram medidas por 4min e expressas como porcentagem do tempo total. No experimento 2, os animais foram separados em dois grupos: Sem Multitarefas (ST) e Multitarefas (MT). O grupo MT foi submetido no intervalo entre treino e teste às tarefas adicionais de Reconhecimento de Objetos e Labirinto Aquático de Morris, enquanto o grupo ST não passou por nenhuma experiência nova. Após a conclusão do experimento, realizamos um teste motor no Campo Aberto e de ansiedade no Labirinto em Cruz Elevado. Foi utilizado o teste t no experimento 1 e ANOVA de uma via com Post Hoc de Tukey no experimento 2. Os resultados estão expressos como média ± erro padrão. N = 6-13 por grupo.

Resultados: No experimento 1, houve diferença significativa entre os grupos TFS (64,3 ± 8,4) e Muscimol (31,5 ± 5,5) quando o teste foi realizado 1 dia após o treino ($p<0,05$). Também encontramos diferença entre TFS (56,2 ± 3,6) e Muscimol (33,3 ± 5,9) no teste feito em 35 dias ($p=0,006$). Porém, não houve nenhuma diferença entre TFS (49,7 ± 6,1) e Muscimol (44,7 ± 6,9) no teste feito em 46 dias ($p=0,6$). No experimento 2, houve diferença entre os animais que receberam TFS (63,4 ± 3,5) e Muscimol (38,4 ± 6,7) no grupo ST ($p=0,02$). Porém, não houve nenhuma diferença entre TFS (56,5 ± 4,5) e Muscimol (52,8 ± 6,5) no grupo MT ($p=0,97$). Além do mais, os animais do grupo MT aprenderam efetivamente as tarefas adicionais a que foram expostos, e não demonstraram nenhuma alteração motora, exploratória ou de ansiedade.

Conclusões: A memória de medo condicionado permaneceu dependente do hipocampo por cerca de 40 dias. No entanto, quando os animais foram expostos a aprendizados adicionais durante esse período, essa mesma memória já era independente do hipocampo 20 dias depois do aprendizado. Com esses resultados podemos inferir que a aquisição de novos aprendizados provavelmente acelera a consolidação sistêmica do medo condicionado, fazendo com que essa memória "saia" do hipocampo mais cedo. Nossos dados indicam que o processo de reorganização da memória não é estático, e sim um fenômeno dinâmico e plástico que pode ser influenciado por novas experiências.

2. INTRODUÇÃO GERAL

2.1. Consolidação Sistêmica

Fig. A: Théodule Ribot

A idéia de que memórias mais antigas são mais resistentes e memórias recentes são mais vulneráveis a lesões e interferências existe desde o século XIX, com as observações de Ribot de pacientes com danos cerebrais e amnésia (Akers and Frankland, 2009). Desde então, estudos clínicos e experimentais têm corroborado esta visão.

Estudos clínicos mostram que pacientes com lesão na região CA1 do hipocampo geralmente exibem 1 a 2 anos de amnésia retrógrada, enquanto que lesões nas regiões perirrinal, entorrinal e parahippocampal levam a uma amnésia retrógrada com extensão de décadas. Já alguns pacientes com danos no córtex frontal, parietal, temporal e occipital tiveram prejuízos em recordar episódios autobiográficos mesmo para os seus primeiros anos de vida (Bayley et al., 2003; Squire, 1992).

Usando modelos comportamentais com diversos animais, estudos anteriores tinham demonstrado que lesões ou inativação farmacológica do hipocampo feitas em pequenos intervalos de tempo após o aprendizado prejudicavam a expressão da memória, mas não quando esses intervalos eram mais longos (Clark et al., 2002; Izquierdo et al., 1997; Kim e Fanselow., 1992; Kim et al., 1995; Quillfeldt et al., 1996; Winocur et al., 2001). Abordagens genéticas tiveram resultados semelhantes (Shimizu et al., 2000; Yasuda e Mayford., 2006).

Em um estudo de neuroimagem de Bontempi et al. (1999) se observou que a evocação de uma memória de discriminação espacial 5 dias após o treinamento provoca um aumento da atividade metabólica do hipocampo, enquanto que aos 25 dias a atividade metabólica elevada é encontrada no córtex cingulado anterior, pré-frontal medial e córtex temporal como vemos na figura abaixo:

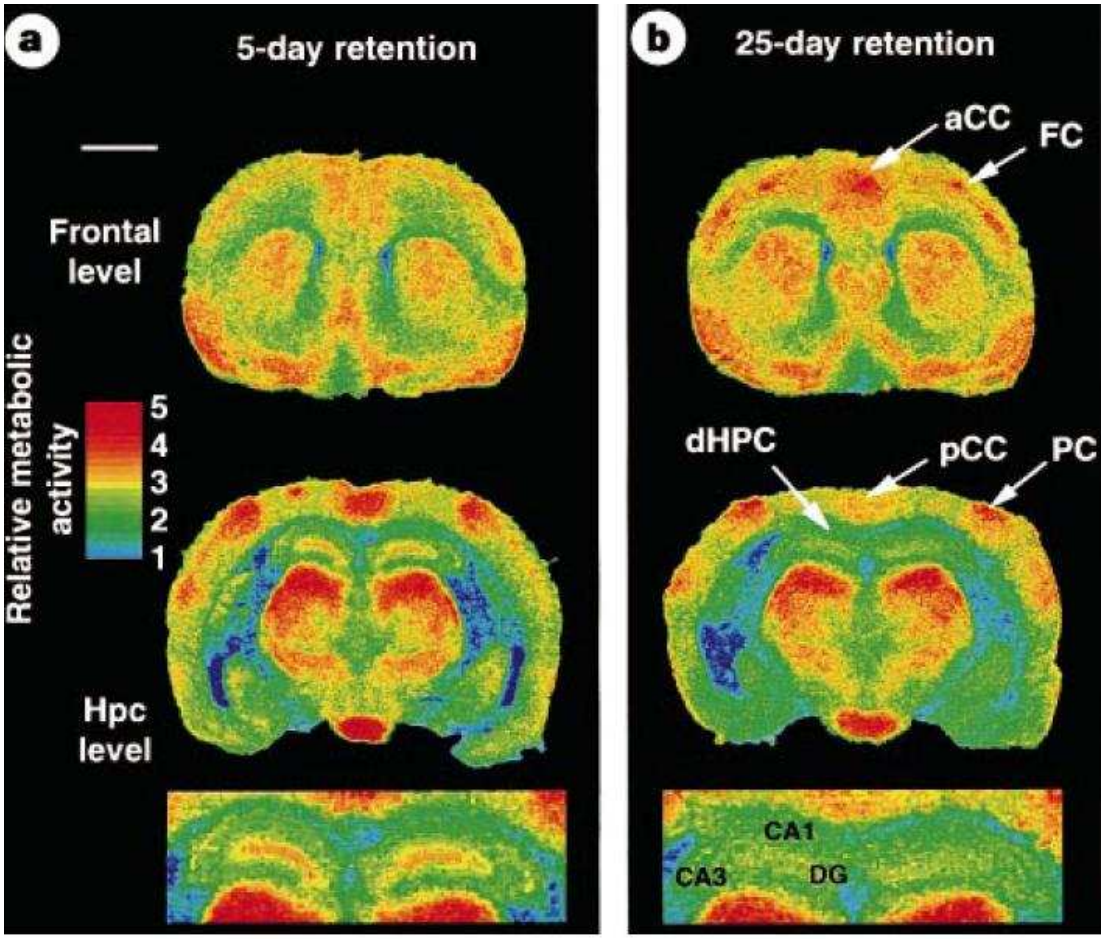

Fig. B: Figura adaptada de Bontempi et. al. (1999) mostrando os resultados das autoradiografias de (14C)2-deoxyglucose obtidas a partir de cortes frontais do encéfalo de ratos que foram treinados em uma tarefa de discriminação espacial por 9 dias e, em seguida, submetidos a testes de retenção 5 dias (a) ou 25 dias (b) após a aquisição inicial. aCC: córtex cingulado anterior; FC: córtex frontal, pCC: córtex cingulado posterior, PC: córtex parietal, dHPC: Hipocampo dorsal.

Em conjunto esses estudos demonstram que memórias recentes e remotas ativam diferencialmente o hipocampo e o neocórtex. Conforme a memória se torna mais antiga, esta conseqüentemente se desliga, se desacopla do hipocampo. Isto é evidenciado pela redução na atividade hipocampal em resposta a evocação de memórias mais remotas e a inabilidade das lesões de afetarem o desempenho nessas tarefas. Em contraste, áreas neocorticais se tornam mais envolvidas conforme a memória "amadurece", o que é evidenciado pelo aumento na atividade cortical em resposta a evocação e o surgimento de efeitos amnésicos causados por lesão (Fig. C).

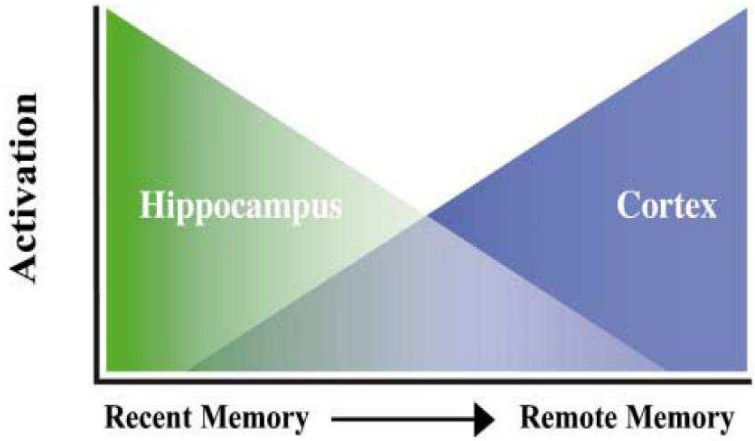

Fig. C: Esquema adaptado de Wiltgen et al. (2004) representando a ativação do Hipocampo e do Neocórtex por memórias recente e remotas.

Esses resultados nos levam a inferir que a memória reorganiza-se com o tempo, tornando-se independente do hipocampo e mais dependente de regiões corticais gradual e progressivamente. Esse processo lento e abrangente a respeito do processamento da memória vem sendo chamado de **Consolidação Sistêmica** ou **Reorganização** da memória (Akers and Frankland, 2009; Bontempi et al, 1999; Debiec et al,2002; Tse and Langston, 2007), ou é simplesmente referido como Consolidação.

Durante essa consolidação, o fortalecimento das conexões córtico-corticais parece ser crucial para as memórias tornarem-se independentes do hipocampo, uma vez que a inibição da plasticidade cortical dificulta a expressão de memórias remotas, mas não de memórias recentes (Frankland et al., 2001). O hipocampo teria um papel fundamental neste processo, com sucessivas rodadas de reativação das conexões hipocampo-corticais, permitindo dessa forma a estabilização e amadurecimento das conexões córtico-corticais. Modelos teóricos propõem que a consolidação cortical deve ser lenta, caso contrário, a informação nova poderia desestabilizar outras já armazenadas (Marr, 1970, 1971).

Na figura da página seguinte (Fig. D) temos uma representação de um possível mecanismo desse processo adaptado de Wiltgen et al. (2004):

- Sob condições iniciais, tanto as ligações intra-corticais quanto as inter-corticais seriam fracas.

- Durante a aquisição, redes neurais se formariam em diferentes áreas através da plasticidade sináptica (Conexões em verde e azul) e mudanças na excitabilidade intrínseca marcariam os neurônios co-ativados recentemente tanto no hipocampo (células azuis) quanto no córtex (células verdes).

- A repetida atividade hipocampal permitiria o reforço das conexões entre as células corticais "marcadas" e com o passar do tempo, as conexões inter-corticais amadureceriam o suficiente e permitiriam a evocação da memória independentemente do hipocampo.

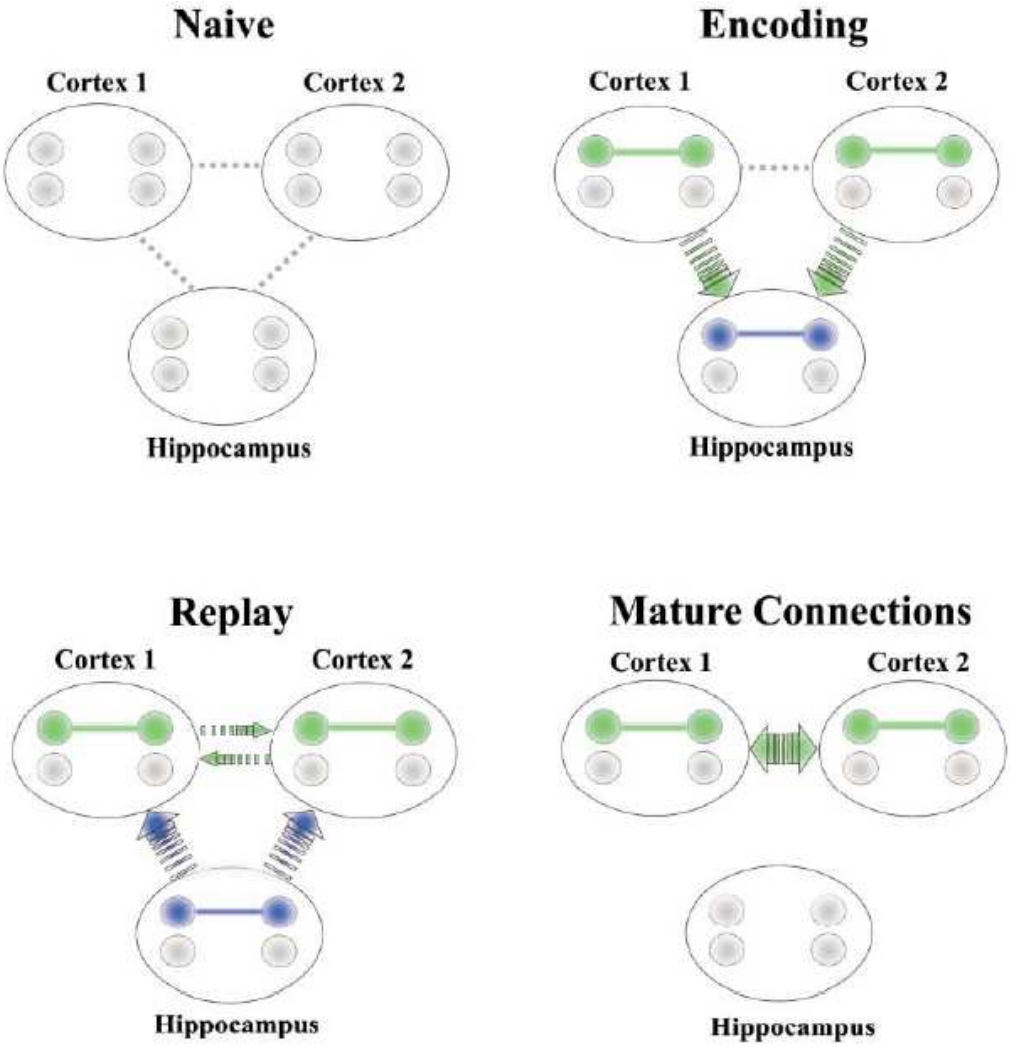

Fig. D: Esquema adaptado de Wiltgen et al. (2004) representando um possível mecanismo da consolidação sistêmica da memória.

Porém, apesar do grande número de evidências demonstrando que o hipocampo possui um papel limitado pelo tempo no armazenamento e evocação de memórias, ainda não é plenamente compreendido como e porque esse processo acontece e quais os parâmetros que poderiam influenciá-lo. Portanto, novos estudos são necessários para aprofundar nossa compreensão acerca dos fenômenos mnemônicos que envolvem o processamento de memórias em longo prazo.

2.2. Hipótese de Trabalho

Uma vez que o hipocampo é uma estrutura indispensável para a codificação inicial de novos aprendizados, mas essencial na expressão dessa memória apenas por um tempo limitado, é válido perguntar por que a memória se torna independente do hipocampo. Uma das razões poderia ser decorrente de que o hipocampo, apesar do seu papel central na codificação de novas memórias, talvez tenha uma capacidade de armazenamento limitada comparada com o neocórtex.

Assim, nossa hipótese é de que informações recém-adquiridas poderiam competir com uma mais antiga para o armazenamento no hipocampo, fazendo com que esta se reorganizasse e se armazenasse em regiões extra-hipocampais mais cedo (Fig. E):

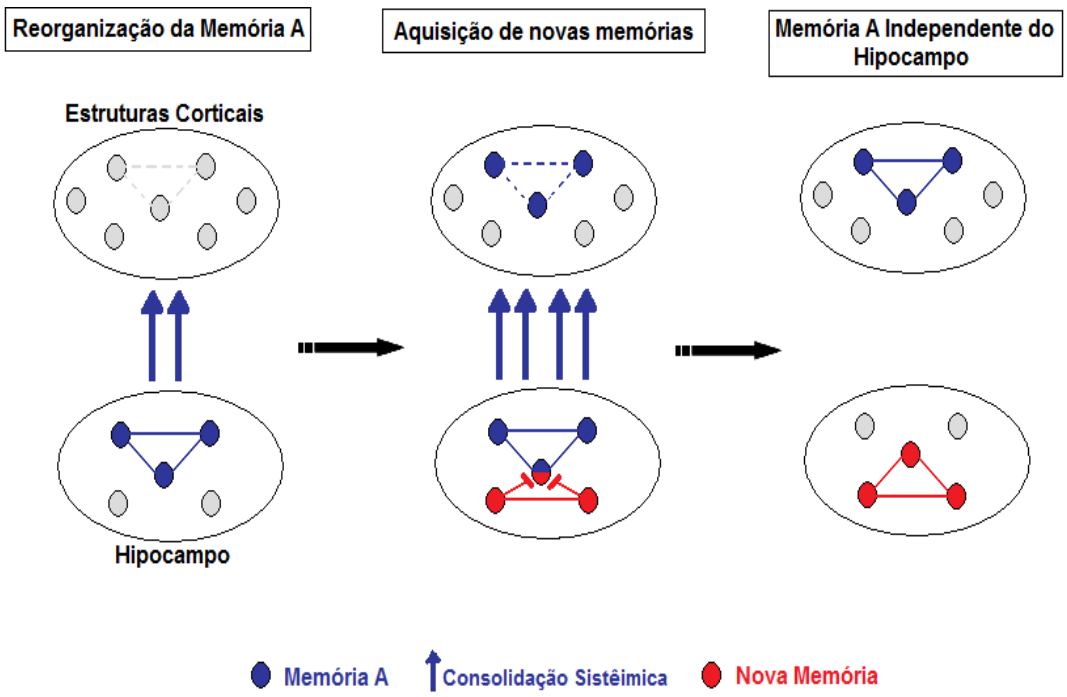

Fig. E: Esquema meramente ilustrativo representando nossa hipótese de trabalho.

2.3. Objetivos

A motivação geral desse trabalho foi então testar a hipótese de que a aquisição de novas memórias poderia acelerar o processo de reorganização de memórias previamente estabelecidas no hipocampo. Assim sendo, os nossos objetivos gerais foram investigar: (a) quanto tempo uma memória de medo condicionado leva para tornar-se independente do hipocampo e (b) quanto tempo essa mesma memória leva para tornar-se independente do hipocampo quando os animais passam por novos aprendizados durante esse período. Para tanto, os objetivos específicos do foram:

- Avaliar o efeito da inativação farmacológica do hipocampo com Muscimol (1ug/lado) sobre a evocação da memória da tarefa de Condicionamento Aversivo ao Contexto (CAC) 1, 35 e 45 dias após o treinamento.

- Avaliar o efeito da inativação farmacológica do hipocampo com Muscimol (1ug/lado) sobre a evocação da memória do CAC 20 dias após o treinamento quando os animais são submetidos, no período entre treino e teste, às tarefas adicionais de Reconhecimento de Objetos e Labirinto Aquático de Morris.

- Verificar se os animais conseguem aprender efetivamente as tarefas adicionais a que são submetidos e, assim, avaliar qualquer alteração cognitiva possível decorrente do elevado número de atividades.

- Avaliar o desempenho dos animais no Campo Aberto e no Labirinto em Cruz elevado e, assim, investigar qualquer alteração motora, exploratória ou de ansiedade decorrente do elevado número de atividades.

3. ARTIGO CIENTÍFICO

Journal Section: Journal of Neuroscience - Behavioral/Systems/Cognitive

Title: New Learning accelerate the system consolidation of a contextual fear memory

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Number of figures: 4

Contents of supplemental material: Supplementary Methods and Figures.

Number of pages: 22

Number of words: 177 (Abstract), 506 (Introduction) and 1183 (Discussion).

Total number of words: 4238.

Keywords: Memory Consolidation, Memory Reorganization, Retrieval, Hippocampus, Fear Conditioning, Muscimol.

Acknowledgements: This research was supported by fellowships and grants from the CAPES (MEC), CNPq (MCT), PROPESQ (UFRGS), and International Foundation for Science (IFS, Stockholm). Authors acknowledge Zelma Regina V. de Almeida for her helpful assistance in the experiments, and Lucas Fürstenau de Oliveira for his contribution on the development of this project.

3.1. Abstract

After initial encoding, memory undergoes a time-dependent reorganization process, by which its retrieval becomes independent of hippocampus and sustained by cortical regions. Despite the great number of evidences showing the time-limited role in memory storage and retrieval of hippocampus, it is still not fully understood how and why it happens and which parameters influence it. At this work, we investigated the influence of new learning experiences upon the system consolidation of a contextual fear memory (CFC). Our experiments showed that the CFC memory takes about 40 days to become independent of hippocampus. However, when animals were exposed to additional learning experiences after CFC training, the retrieval of this memory was already independent of hippocampus 20 days later. The newly acquired information probably accelerated the CFC system consolidation, leading to its reorganization and storage in extra-hippocampal regions earlier. Our data indicate that the reorganization of memory and the process by which its retrieval becomes independent of hippocampus is dynamic and can be influenced by other non-related learning episodes.

3.2. Introduction

The idea that older memories are more resistant and recent ones are more vulnerable to injuries and interferences has been hypothesized since the 19th century with Ribot's observations of patients with brain damages and temporally graded amnesia (Akers and Frankland., 2009). Since then, clinical and experimental studies have been corroborating with this view.

Clinical studies show that patients with localized CA1 lesion usually displays 1 to 2 years retrograde amnesia, while peririnal, enthorinal e parahippocampal lesions lead to retrograde amnesia with an extension of decades. Some patients with damages to frontal, parietal, temporal and occipital cortex had impairments to recall autobiographical episodes even for their early life (Bayley et al., 2003; Squire, 1992).

Using several animal´s behavioral models, previous studies had shown that lesions or pharmacological inactivation of hippocampus made at short delays after training hindered memory expression, but not at longer delays (Clark et al., 2002; Izquierdo et al., 1997; Kim and Fanselow, 1992; Kim et al., 1995; Quillfeldt et al., 1996; Winocur et al., 2001). Genetic approaches had similar results (Shimizu et al., 2000; Yasuda and Mayford, 2006).

Also, a neuroimaging studie of Bontempi et al. (1999) showed that the retrieval of a spatial discrimination task 5 days after training causes an increased hippocampal metabolic activity, while at 25 days after training an elevated metabolic activity is found in anterior cingulated, prefrontal, medial and temporal cortices.

Taken together, these studies clear suggests that memory reorganizes itself in a time-dependent manner, becoming independent of the hippocampus and dependent of cortical regions. This process has been called system consolidation or memory reorganization.

During the consolidation, the strengthening of cortico-cortical connections appears to be crucial for memories to become independent of hippocampus, since the inhibition of cortical plasticity hinders the expression of remote, but not recent memories (Frankland et al., 2001). The hippocampus would have a key role in this process, as successive rounds of reactivation of hippocampal-cortical connections would precede the cortico-cortical stable connections (Wiltgen et. al., 2004). Theoretical models propose that cortical consolidation must be slow, otherwise new informations would destabilize those already stored (Marr, 1970, 1971).

Some recent studies brought up interesting evidences indicating that memory system consolidation is not a static, but a dynamic process. Extensive experience in a context or in a given task enables fast cortex consolidation and memory´s survival after hippocampal lesion (Lehman et al., 2009; Winocur et al., 2005). Also, once memories are cortically established, neuronal circuits can create mental schemas making possible for new similar memories to become rapidly independent of the hippocampus (Tse et al., 2007).

Since the hippocampus is a structure necessary for memory initial encoding, but only essential for retrieval during a limited time, it is valid to ask why memory become hippocampus-independent. One possibility may be that the hippocampus, despite its central role in memory encoding, may have a limited storage capacity.

We hypothesize that newly acquired informations would compete with older ones for hippocampus housing, leading to a faster reorganization and atorage in extra-hippocampal regions. To address this issue, first we attempt to identify how much time is necessary for a contextual memory to become hippocampus independent (Experiment 1) and so we investigated if the acquisition of new memories during this period could interfere with this process (Experiment 2).

3.3. Material and Methods

Male Wistar rats were submitted to a Contextual Fear Conditioning (CFC) protocol. In the conditioning trial (training), animals were placed in the chamber for 3 min and received 2 X 2 sec footshocks of 0.7 mA separated by a 30 sec interval and kept in the conditioning environment for an additional minute. The test session takes place 1, 35 or 45 days after training in Experiment 1, or 20 days later in Experiment 2, where animals were re-exposed to the same context for 4 min and the freezing behavior was registered and usedemployed as a memory index.

In order to investigate whether the hippocampus was necessary or not for the retrieval of this contextual memory, rats of both experiments were submitted to a stereotaxic surgery and 15 min before the test session, a classical amnesic drug (Holt and Maren, 1999) or its vehicle was infused into the dorsal hippocampus (Fig. S1). The surgeries took place five days before the CFC test, when all animals were bilaterally implanted with cannulae aimed at the CA1 area of the dorsal hippocampus, according to Paxinos and Watson (2007). Animals that haven't correct cannulae placements were discarded in the statistical analysis. The drug used in the infusions into hippocampus was the Muscimol, a selective GABA agonist (1 ul/side; Tocris Cookson Inc., Ellisville, MO, USA), or its vehicle, TFS (phosphate-buffered saline with 8% dimethylsulfoxide).

Animals of Experiment 2 were divided into two experimental groups: Single-task (ST) and Multiple-tasks (MT) (Fig. 1). ST group did not pass to any different experience between the training and test of CFC. At the same period, however, the MT group was trained into two additional tasks: Object Recognition (OR) and

Morris Water Maze (WM). If an increase in mnemonic activity of hippocampus can really accelerate the corticalization of a previous memory, we expect that in the MT group the CFC memory will be independent of hippocampus more quickly. For more details, see supplementary material.

To ensure that the elevated number of activities and the major manipulation of animals on the MT group didn't affect the motility and anxiety behavior, we performed the Open Field (OF) and Elevated plus-maze (EPM) tasks one day after the end of experiments.

3.4. Results

CFC memory takes about 40 days to become hippocampus-independent.

In experiment 1, there was a significant difference between Vehicle and Muscimol when the test was performed 1 and 35 days after training ($p<0,01$; T Test), but not 45 days later ($p=0,6$; T Test) (Fig. 2a). This experiment shows that retrieval of the contextual fear memory can be dependent of hippocampus for at last 35 days after the learning session.

New learning makes CFC memory hippocampus-independent in 20 days.

In experiment 2, the animals of ST group that received Muscimol froze significantly less compared to the ones that received only Vehicle, TFS ($p= 0,02$; Tukey's multiple range test). So, the injection of this nervous system depressor, Muscimol, into hippocampus impaired the contextual memory retrieval in this group (Fig. 2b).

In the MT group, however, there wasn't any difference between groups that received the amnesic drug, muscimol, and it's vehicle ($p= 0,97$; Tukey's multiple range test). Thus, in this group that was exposed to additional tasks, the muscimol administered into hippocampus was not amnesic (Fig. 2b).

This experiment corroborates that retrieval of the contextual fear memory is still dependent of hippocampus even twenty days after the training session, as we can find in the literature (Bianchin et al., 1993). Nevertheless, when the animals were exposed to additional learning after the CFC training, the retrieval of this contextual

memory was already independent of hippocampus twenty days after the memory acquisition.

On MT group, animals also effectively learned all the additional tasks that they were submitted. On the OR test, that was significantly difference between novel and familiar objects exploration (p= 0,000; Paired Samples Test), as shown on Fig. 3a. In addition, there wasn't any preference for objects on the training day (Fig. S2). The time taken to find the platform in the last day of WM training was significantly lower compared to the first one (p=0,000; Paired Samples Test), as we can see on Fig. 3b. On the WM test, the animals significantly explored more the Target Quadrant than the Opposite one (p= 0,001; Paired Samples Test) (Fig. 3c). This results show that the great number of activities in this group, and possible stress, didn't affect the cognitive capacity of the subjects.

The number of crossings and rearings on the OF was used to analyze the exploratory behavior of rats (Fig. 4a and 4b). Independent T Test showed no difference between groups in the number of crossings (p=0,7) as well in the number of rearings (p=0,9). The time spent in the open arms on the EPM was used to evaluate anxiety-like behavior. Independent T Test showed no differences in the time spent in the open arms between groups (p=0,9) as displayed in Fig. 4c. Therefore, the increased number of activities and the higher level of manipulation of the MT animals didn't affect their exploratory activity neither their anxiety state. Additional statistics also showed that the earlier Muscimol injection, in the CFC Test, didn't cause any lasting pharmacological effect or damage that could interfere with the motility or anxiety behavior of these animals (Figs. S3 and S4).

3.5. Discussion

At this study we tested if new leanings during the system consolidation of a contextual fear memory could interfere with this processes. As main task to investigate the hippocampal participation in retrieval we used the contextual fear conditioning. We chose this task because it is a hippocampus dependent and it is easily learned in a single training session. Moreover, hippocampus dependency of contextual fear conditioning memory across time has been well studied (Debiec et al., 2002; Kim and Fanselow, 1992; Maren et al., 1997).

To propitiate the new experiences WM and OR tasks were chosen, for three major reasons: (i) both are hippocampus dependent (Redish & Touretzky, 1998; Kelly et al. 2003); (ii) their acquisition require many sessions, maximizing the requirement of hippocampus during the period of CFC systems consolidation; and (iii) this tasks do not involve shock neither food restriction, minimizing the stress of animals. Although there are some discussion if OR is hippocampus-dependent or not (Langston & Wood, 2009), we were based on protocols that was demonstrate to requires hippocampus activity and protein synthesis (Rossato et al., 2007; Langston & Wood, 2009). Anyway, the two habituations that are part of the OR protocol classically requires hippocampus (Izquierdo & Medina, 1995; Squire, 1992). These characteristics allowed us to test our hypothesis.

During training and test sessions we tried to minimize stress at maximum. Stress and fatigue was a constant concern, as our goal was to verify only the mnemonic effect of the additional tasks, what could be disguised by higher anxiety and lower mobility. The multi-task protocol, however, didn't interfere in the EPM and OF performance.

In this study no attempt was made to separate the influence of WM and OR and our protocol doesn't permit to evaluate if both additional tasks were responsible for our findings or only one of them.

We found that pre-test administration of muscimol induces profound retrograde amnesia, as previously reported (Holt and Maren, 1999), 1 and 35 days after training, and has no effect when administered 45 days after learning. However, with additional learning the fear conditioning's memory was spared. As memory maintenance is dependent of different structures over time, with hippocampus playing a major role in early stages but not in remote points (Frankland and Bontempi, 2005; Frankland et al., 2007; Wiltgen et al., 2004), the preserved fear memory of MT group after muscimol treatment suggests that this memory has already become independent of hippocampus and is probably been sustained by parahippocampal or cortical structures. The additional learnings, which occurred after the training session of the contextual fear conditioning, somehow accelerated memories reorganization process. This influence was purely mnemonic since exploratory and anxiety-like behaviors were unaffected.

Recent studies have also demonstrated some circumstances that can influence the system consolidation. Tse & Langston (2007) elegantly reported that this process can occur extremely quick if an associative "schema", into which new information is incorporated, has previously been created. Winocur et. al (2005) demonstrated that extensive and long experience in a same environment leads to spatial representations that are independent of hippocampus. Lehmann et. al. (2009) showed that a very strong training with numerous trials on CFC can make this memory rapidly

independent of hippocampus. All these studies, unlike ours, influenced the corticalization rate of memories by reinforcing the same learning or environment, and acting before the acquisition of the studied memory. Here we report, for the first time, that new learning after a fear memory acquisition can influence its reorganization rate.

Since the amount of post-training experience was the differential factor between the groups in this study, and that this experiences lead to learning and memory storage, our major conclusion is that the amount of encoded information somehow influenced and accelerated memory reorganization from hippocampus to other structures. We showed that one memory systems consolidation is affected by the consolidation of other dissimilar memories.

We suggest that the additional learnings lead to a higher hippocampal activity and a faster clearance of the fear conditioning's memory, with a parallel faster consolidation in cortex, avoiding hippocampus overloading and making possible to new memories be acquired and consolidated. Faster hippocampal clearance might happen due increased neurogenesis (Tsient et al., 2001), as neurogenesis appears to be regulated by experience (Gould et al., 1999; Greenough et al., 1999)

During memory consolidation, the hippocampus activates neurons in cortical areas (Qin et al., 1997), what could allow to different cortical modules to be reactivated together, strengthening connections between them. This is a proposed mechanism, called synaptic reentry reinforcement, by wich memories would turn hippocampus independent through the strengthening of cortico-cortical connections (Shimizu et al.,

2000; Wang et al., 2006). Hippocampal elevated activity may also lead to an enhanced synaptic reentry reinforcement, accelerating cortical consolidation and hippocampus-independency observed in our results.

Accordingly, hippocampus main function might be, besides encoding, to storage memories fast, but just while they are not stabilized in cortex. After it, previously occupied hippocampal networks would be disengaged from the preceding memory (partially or completely) and would be ready again for new memories. Thus, as more the hippocampus is required, more rapidly memories stored in this structure would be disengaged from it.

Some theoretical models propose that the hippocampus serves as an "index" or "pointer" to cortically encoded information, guiding the slow systems-level consolidation process that is thought to involve hippocampal-neocortical interactions over time (Teylor and DiScenna, 1986; Siapas and Wilson, 1998). So, another possible reason for the system consolidation acceleration by new learnings is that it happened as a simple consequence of cortico-hippocampus interaction enhancement caused by the hippocampus increased activity. These explanations are not exclusive, and could act together to reach the same result, allowing the faster memory disengagement of hippocampus.

In a great example of how the synaptic consolidation of a memory trace can influence another, Ballarini et al. (2009) submitted animals to two different tasks, one that can induce LTM and another that just induced STM. When these ones were pared, at specific time window, the weak experience used the proteins synthesized

for the strong one by memory tags (Frey & Morris, 1997), and produced effectively LTM. Those mechanisms that enabled this phenomenon could be occurring in a broader level, as the system consolidation of memory. If it is true, we can speculate that similar mechanisms could be contributing to the additional tasks interference on the systems consolidation of CFC trace in our experiment.

In conclusion, we found for the fist time that new learning experience can accelerate the systems consolidation of a contextual fear memory, making it hippocampus-independent earlier. It's possible that this observed phenomenon occurs in order to disengage this memory faster and thus "release space" on hippocampus to the newly acquired information. Thinking in that way, the amount of hippocampus requirement can influence the reorganization rate of a contextual memory and the consequent storage on neocortical structures. These findings have implications for the neurobiology of learning and memory, indicating that the reorganization of memory and the process by witch its retrieval becomes independent of hippocampus is dynamic and can be influenced by other non-related learning episodes. In consequence, the time course for systems consolidation of a given memory can be different depending of the circumstances on which it occurs.

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3.7. Figures and Legends

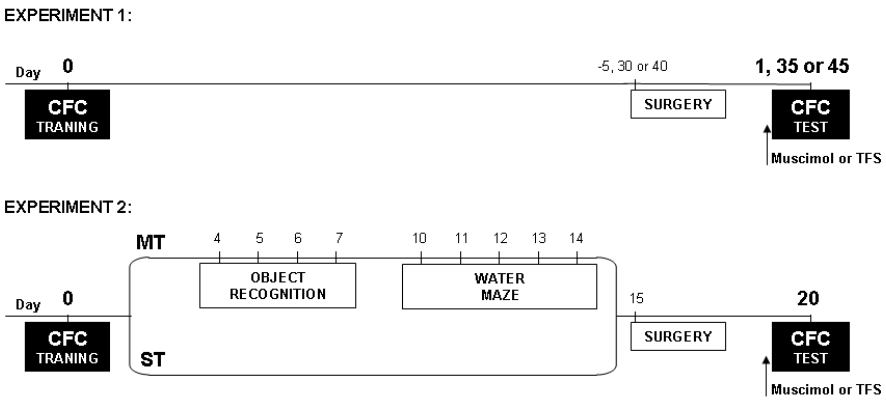

Figure 1 - Protocol schema of experiments. On day 0, all animals were trained on the Contextual Fear conditioning (CFC) and tested 1, 35 or 45 days later in Experiment 1 and 20 days later in Experiment 2. In experiment 2, animals were divided into two groups: Single-task (ST) and Multiple-tasks (MT). The animals of the MT group, on day 4 and 5, were habituated to the experimental arena of Object Recognition for 30 min. On day 6, each rat was placed in the arena containing two objects and left to freely explore them for 5 min. On day 7, the test phase was performed, where one of the objects was exchanged for a novel. On days 10 to 13, animals were submitted to the WM task. Rats were subjected to four training days (sessions), each session consisting of four trials, and to a probe trial on the 14th day. On day 15, the surgeries were made in all groups, for the bilaterally implantation of cannulae in the hippocampus. Finally, on day 20, the groups were submitted to the CFC Test, when, 15min before received an intrahippocampal injection of Muscimol or its Vehicle.

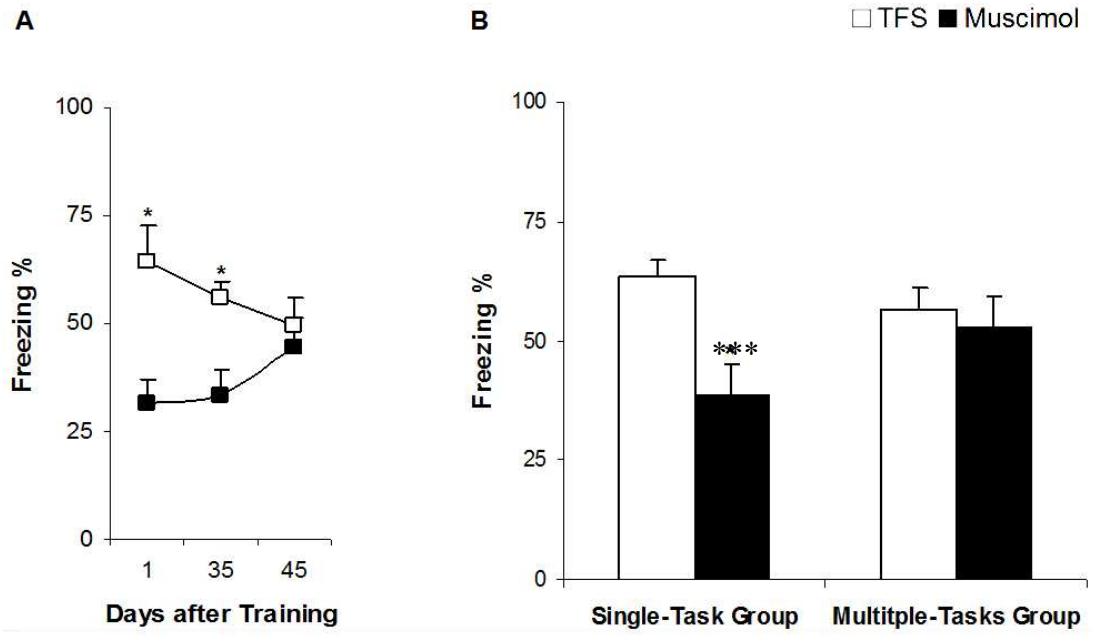

Figure 2 - Effect of intrahippocampal infusion of Muscimol (1ug/side) upon Contextual Fear Conditioning test session, performed 1, 35, 45 (A) or 20 (B) days after training. Data expressed as Mean $\pm$ S.E.M. of percentual freezing time in a 4-min test session. * Significant difference between TFS and Muscimol 1 and 35 days after training ($P<0,05$; Independent T Test). *** Significant difference between TFS and Muscimol of the Single-task group ($P= 0,02$; Tukey's multiple range test). There were no significant difference between groups 45 after training. Also, there were no significant difference between Vehicle and Muscimol of the Multiple-tasks group ($P= 0.97$, Tukey's multiple range test). Moreover, no difference was find between TFS/ST and TFS/MT groups ($P= 0,84$; Tukey's multiple range test). N= 10 and 12

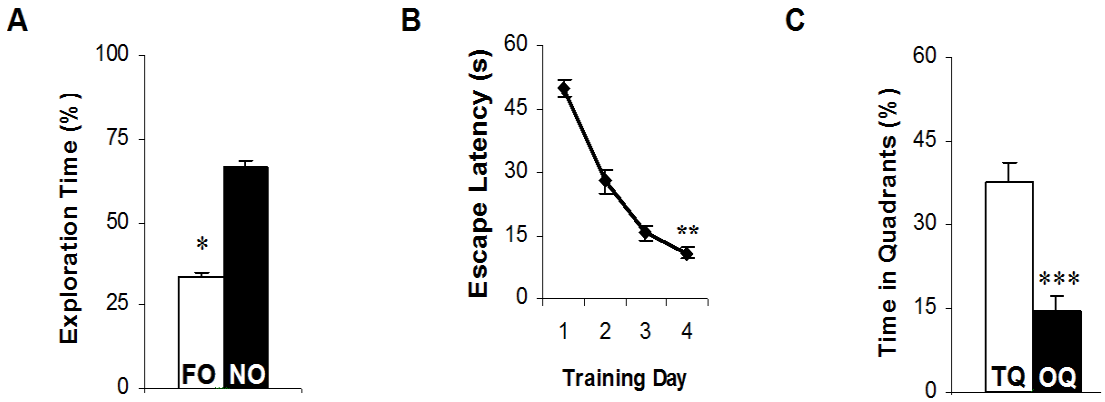

Figure 3 - Performance of the MT group in the additional tasks. Data expressed as Mean ± S.E.M. N= 23. **A**. RO test session, showing percentage of time exploring a particular object over the total time of object exploration. B. Mean escape latency (s) during WM training sessions. C. WM test, showing the time (s) spent in target (TQ) and opposite (OQ) quadrants during the 60 seconds of exposure. * Significant difference between novel (NO) and familiar (FO) objects in the RO test session (p= 0,000; Paired Samples Test). ** Significant difference between the escape latency time of the first and the fourth day of training (Paired Samples Test). *** Significant difference between quadrants (p= 0,001; Paired Samples Test).

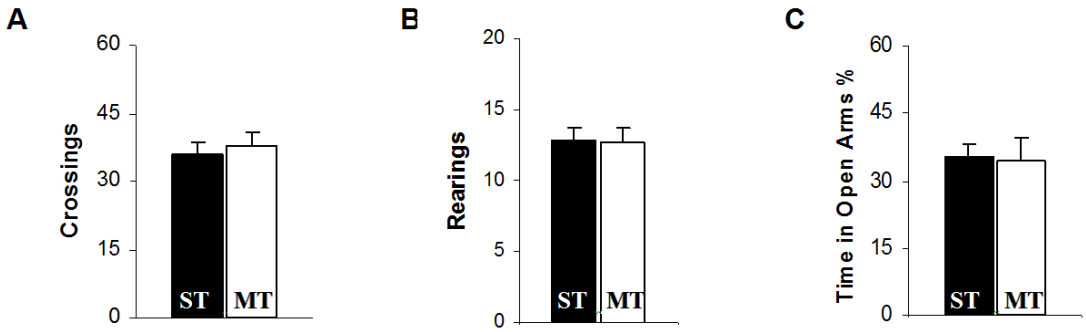

Figure 4 – Performance of animals on the motility and anxiety tests. Data expressed as Mean ± S.E.M. N= 20-23 per group. **A and B.** Number of crossings and rearings in the Open Field during 3min of exploration. **C.** Percentual of time spent in the open arms during the 3-min exposure to the elevated Plus Maze. There were no significant differences between groups in all tests (Independent T Test, p> 0,05).

3.8. Supplementary Material

Suplementary Methods

Animals. Fifty Wistar rats (age 2–3 months, weight 250–300 g) from our breeding colony were used in these experiments. Animals were housed in plastic cages, four to five in a cage, under a 12 h light/dark cycle and at a constant temperature of 24±1 °C, with water and food *ad libitum*.

Contextual Fear Conditioning (CFC). The conditioning chamber consisted in an illuminated Plexiglas box (25.0×25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart). In the conditioning trial (training), rats were placed in the chamber for 3 min and have received 2 X 2 sec footshocks either of 0.7 mA separated by a 30 sec interval and kept in the conditioning environment for an additional minute. Twenty days later, the test session takes place, where the animals were re-exposed to the same context for 4 minutes and the freezing behavior (complete lack of movement, except for respiration) was registered in percentage of time session by an experienced observer (blind to the treatment conditions) end employed as a memory index.

Stereotaxic surgery and cannulae placement. Five days before the CFC test, all animals were anesthetized by a mixture of ketamine and xylazine (i.p., 75 and 10 mg/kg, respectively) and bilaterally implanted with a 27-gauge guide cannulae aimed at AP −4.2 mm (from bregma), LL +3.0 mm, DV 1.8 mm, just 1.0 mm above the CA1 area of the dorsal hippocampus (according to Paxinos & Watson, 2007). After the experiments all subjects were sacrificed and had their brains dissected and

preserved in 10% formaldehyde for a later cannulae position check under low magnification. Animals that haven't correct cannulae placements weren't included in the statistical analysis.

Intrahippocampally infused drugs. Fifty minutes before the CFC test session, a 30-gauge infusion needle was fitted into the guide cannulae, with its tip protruding 1.0 mm beyond the guide cannula end and aimed at the pyramidal cell layer of CA1 of the dorsal hippocampus. A volume of 1 μl was bilaterally infused at 40 μl/h rate. Animals were divided into two groups, each receiving one of these drugs: Muscimol, a selective GABA agonist (1 ul/side; Tocris Cookson Inc., Ellisville, MO, USA), or its vehicle (phosphate-buffered saline with 8% dimethylsulfoxide).

Object Recognition. Three days after de training in CFC, the object recognition task was conducted in an open field arena (x cm) built of transparent acrylic. The open arena and the objects were cleaned thoroughly between trials to ensure the absence of olfactory cues. Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. The time spent exploring each object was recorded by an experience observer and expressed as a percentage of the total exploration time in seconds. On day 1 and 2, the animals were habituated to the experimental arena by allowing them to freely explore it for 30 min in the absence of stimulus objects. On day 3, rats were placed in the arena containing two objects and left to freely explore them for 5 min. At the day 4, the test phase was performed, where one of the objects was randomly exchanged for a novel object, and rats explore than for 5 min too. If rats remeber the familiar object, they will direct more exploration at the novel. Student's *t*-test was used to analyze the data.

Morris Water Maze. After two days of the OR task, rats were submitted to the Morris water maze. The maze consisted of a black circular pool with 180 cm in diameter filled with water (temperature around 23 °C, depth 40 cm) situated in a room with visual cues on the walls. A transparent platform with 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided in four quadrants and had four points designed as starting positions (N, S, W or E). Rats were subjected to four training days (sessions) and a probe trial in the 5th day. Each training session consisted of four trials with a 10 min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied in every session and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeeded, it was gently guided to the platform and left on it for 20 s. The probe trial consisted of a single trial, with the platform removed. Here, the time spent in the target, as well as in the opposite quadrants, were measured and used as an index of memory for the task (Pereira, *et al.,* 2006).

Open Field (OF) and Elevated plus-maze (EPM): To be sure that the elevated number of activities and the major manipulation of the animals on the MT group didn't affect the motility and anxiety of the rats, in the end of all experiments we performed the Open Field and Eleveted plus-maze tasks. Open Field was studied using a 50 cm high, 60x40 cm plywood box with a frontal glass wall and a linoleum Xoor divided in 12 equal rectangles. Animals were left there for 3 min and the number of rearings and crossings between sectors were counted. The EPM was conducted using a standard plus maze apparatus kept 80 cm above the floor,

consisting of four arms arranged in the shape of a plus sign (arms measured 50 ×10 cm). Two of the arms, opposite to each other, were surrounded by a 1 cm high Plexiglas ledge (open arms), and two other arms (closed arms) presented a 40-cm height wall. The behavioral test was conducted using red light illumination. The animal was placed in the center of the plus maze and remained in the apparatus for 3 min. The time spent in the open or enclosed arms were analyzed and expressed as the time spent in the open arms/time spent in all X 100. This parameter is considered to reflect the "anxiety" level experienced by the animal.

Supplementary Figures

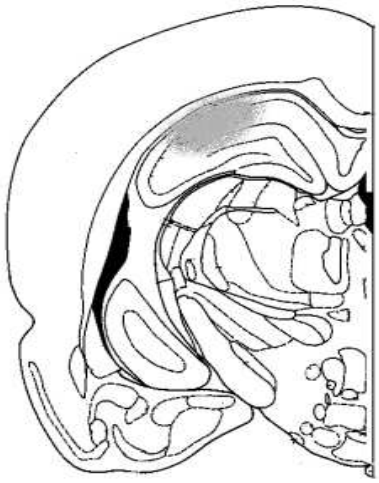

Figure S1: Schematic drawing of rat brain section at plane A -4.3 of the atlas of Paxinos and Watson (1986), showing in *stippling*, the extension of the area reached by the infusions in the dorsal hippocampus.

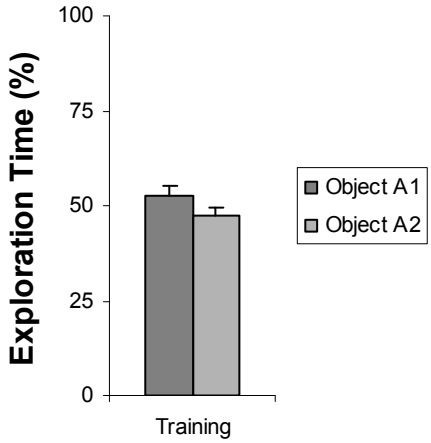

Figure S2: RO training session of the Multiple-Tasks group, showing percentage of time exploring a particular object over the total time of object exploration. There was no preference for the objects (p=0,3; Paired Samples Test). N=23.

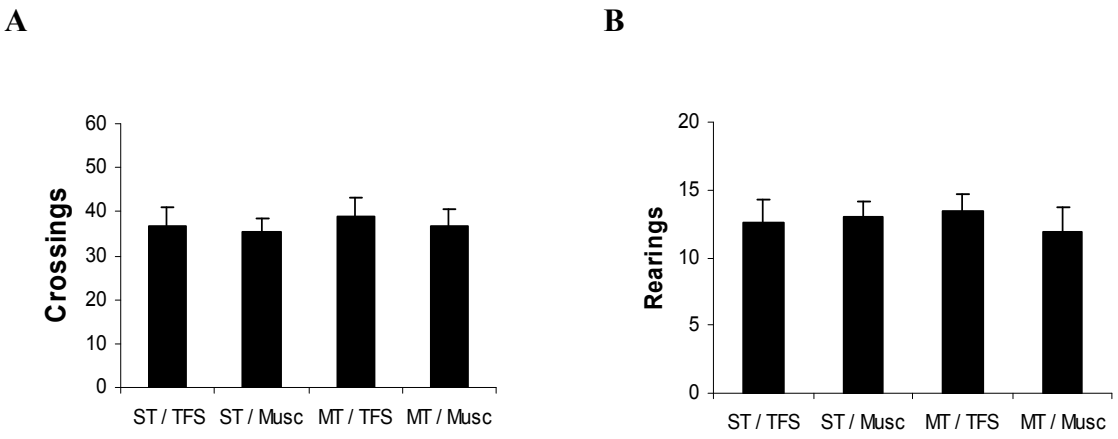

Figure S3: Performance of animals on the motility test, comparing the Multiple-Tasks (MT) and Single-Task (ST) groups, as well the subgroups of each one, that received TFS or Muscimol on the CFC Test. Data expressed as Mean ± S.E.M. N= 10-12 per group. **A and B.** Number of crossings and rearings in the Open Field during 3min of exploration. There was no difference between groups (One-Way ANOVA, p> 0,05).

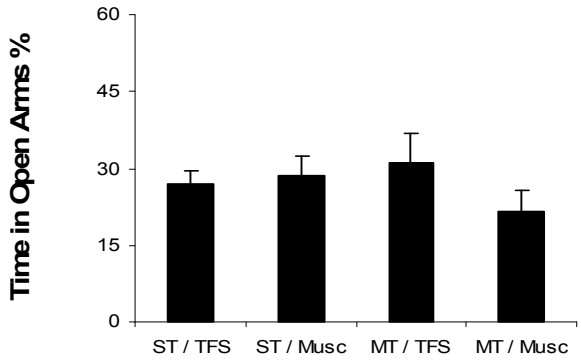

Figure S4: Performance of animals on the anxiety test, comparing Multiple-Tasks (MT) and Single-Task (ST) groups, as well the subgroups of each one, that received TFS or Muscimol on the CFC Test. Data expressed as Mean ± S.E.M. N= 10-12 per group. Percentual of time spent in the open arms during the 3-min exposure to the elevated Plus Maze There was no significant difference between groups in all tests (One-Way ANOVA, p> 0,05).

4. CONSIDERAÇÕES FINAIS

4.1. Conclusões

De acordo com os objetivos traçados nesse trabalho obtivemos resultados que nos permitem concluir especificamente que:

- A inativação farmacológica do hipocampo com Muscimol (1ug/lado) foi amnésica sobre a evocação da memória da tarefa de Condicionamento Aversivo ao Contexto (CAC) aos 1 e 35 dias após o treinamento, mas não teve nenhum efeito aos 45 dias.

- A inativação farmacológica do hipocampo com Muscimol (1ug/lado) sobre a evocação da memória do CAC 20 dias após o treinamento não teve nenhum efeito nos animais que foram submetidos, no período entre treino e teste, às tarefas adicionais de Reconhecimento de Objetos e Labirinto Aquático.

- Os animais conseguiram aprender efetivamente as tarefas adicionais a que foram submetidos demonstrando que não houve alteração cognitiva decorrente do elevado número de atividades.

- Os animais que passaram por essas atividades adicionais apresentaram desempenho igual ao do grupo controle no Campo Aberto e no Labirinto em Cruz Elevado, demonstrando que não houve qualquer alteração motora, exploratória ou de ansiedade decorrente do elevado número de atividades.

Essas conclusões empíricas mostram que, neste trabalho: (a) a memória de medo condicionado levou cerca de 40 dias para tornar-se independente do hipocampo em animais que não passaram por nenhuma experiência nova enquanto que (b) quando os animais foram expostos a aprendizados adicionais durante esse período, essa mesma memória tornou-se independente do hipocampo em apenas 20 dias.

Podemos assim inferir que a aquisição de novas memórias provavelmente acelerou a consolidação sistêmica do medo condicionado, fazendo com que esse traço "saísse" do hipocampo mais cedo. Percebemos então que o processo de reorganização da memória não é estático, e sim um fenômeno dinâmico e plástico que pode ser influenciado por novas experiências.

As conclusões obtidas corroboram com a hipótese inicial de que a aquisição de novas memórias poderia acelerar o processo de reorganização de memórias previamente estabelecidas no hipocampo. Porém, mais experimentos são necessários para apoiar e embasar mais solidamente a hipótese apresentada.

4.2. Perspectivas

As respostas encontradas levantam novas questões e ajudam a formular novos experimentos que irão aprofundar a nossa compreensão acerca do fenômeno observado neste trabalho. Dando continuidade a esse projeto então, nossos objetivos futuros são:

- Investigar quanto tempo uma memória de medo condicionado leva para estabecer-se em diferentes estruturas corticais, como o Córtex Cingulado Anterior (CCA).

- Investigar quanto tempo essa mesma memória leva para estabelecer-se em estruturas corticais (CCA) em animais que passam por novos aprendizados durante esse período.

- Quantificar a expressão de indicadores de atividade neural, como os Genes de Expressão Imediata c-fos e zif no Hipocampo e no CCA, em resposta a evocação da memória de medo condicionado 1, 35 e 45 dias após o treino.

- Quantificar a expressão de indicadores de atividade neural no Hipocampo e no CCA em resposta a evocação dessa mesma memória 20 dias após o treino em animais que passam por novos aprendizados durante esse período.

- Replicar os resultados mais importantes desse trabalho com outra tarefa comportamental, como a Esquiva Inibitória.